EXHIBIT 19

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Anticomplementary Activity of Human Immunoglobulin G

I. Mechanism of the Artifactual Increase in Anticomplementary Activity of IgG During the Assay

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Abstract. The anticomplementary activity of IgG can be increased up to 20-fold by pipetting during the preparation of serial dilutions for the assay of this activity. Albumin, if added to the IgG solution before the serial dilutions, completely prevents this artifactual increase in activity. Polyethylene glycol, polyvinyl pyrrolidone, methyl cellulose, gelatin and octanol are also effective stabilizers of IgG.

Repeated pipetting of IgG solutions caused marked linear increase in their anticomplementary activity. The formed anticomplementary activity was due to small amounts of highly aggregated protein. The amount of activity formed depended on at least four factors: [1] the number of pipetting steps; [2] the IgG concentration; [3] the level of albumin or other stabilizers, and [4] the pH, which influences the stabilization by albumin.

The anticomplementary activity of IgG is increased up to 100-fold by exposure to gas-liquid, liquid-liquid and hydrophobic solid-liquid interfaces. Albumin and polyethylene glycol prevent the activity increase during these treatments. The tendency of IgG to aggregate at interfaces and the ability of albumin and other substances to prevent the aggregation paralleled their rate of adsorption to the air-water interface. Solutions of dansyl chloride in decane emulsified in aqueous solutions of IgG and albumin specifically label the proteins at the liquid-liquid interfaces. The mechanism of stabilization can be explained by preferential adsorption of surface-active proteins and polymers.

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Introduction

Many investigators have documented that isolated IgG has marked anticomplementary properties [11, 12, 28]. The anticomplementary components in IgG preparations have been shown to be aggregates [10, 15, 16, 18, 19, 32] which form spontaneously or as a result of the isolation procedures, and which can also be generated by heating [15, 18, 19], chemical coupling [19] or exposure to organic solvents [33].

The presence of anticomplementary aggregates limits the clinical applications of immunoglobulin preparations. Intravenous administration of such preparations can cause serious systemic reactions [1]. Intramuscular injections, while being better tolerated, are in many indications significantly less effective. Various treatments (e. g., low pH, proteolysis, chemical modification) have been used to abolish the anticomplementary properties of IgG and render it safe for intravenous administration. However, the treated preparations generally have decreased stability or lessened biological effectiveness. These shortcomings have prompted the search for ways to prevent or control the aggregation of IgG during its isolation and storage.

DAVIS *et al.* [11] reported in 1944 that albumin and other serum fractions decrease or abolish the anticomplementary activity of IgG. They assumed that albumin somehow inhibits the inherent anticomplementary properties of IgG. The mechanism of this inhibition remained obscure. Recently, in what appeared to be an unrelated observation MALGRAS *et al.* [24] reported that serial dilution of IgG solutions sometimes caused increased anticomplementary activity and gave falsely high results when used in assays for this activity.

In routine measurements of anticomplementary activity in this laboratory, we observed unusual patterns of complement fixation with some IgG samples. IgG which had been treated at pH 4 was not anticomplementary at high concentrations but showed sharply increased anticomplementary activity when serially diluted more than 1 : 32. When monomeric IgG obtained by gel filtration was tested, the undiluted solution was not anticomplementary, but 1 : 2, 1 : 4 and 1 : 8 serial dilutions of it completely inhibited added complement. The present investigations were undertaken to find the reason for these anomalous complement binding patterns. The results indicated that pipetting of IgG serial dilutions during the assay causes a marked increase in the anticomplementary activity and that this increase is prevented by albumin and a variety of other substances. The results of an extensive

study of the effect of pipetting on IgG solutions are reported. Furthermore the effect of exposure of IgG to interfaces and the influence of surface active substances were studied.

Materials and Methods

Proteins. γ-globulin fraction II analogue was obtained from human plasma by the Cohn cold ethanol fractionation as modified by KISTLER and NITSCHMANN [21]. Lyophilized powder was dissolved in water as a 12- to 14-percent solution, sterile-filtered and stored at −20°C or +4°C. These preparations, further designated as fraction II, were found to consist of 96–98% γ-globulins, 2–3% albumin and traces of α- and β-globulins when analyzed by zone electrophoresis on cellulose acetate. Upon micro-immunoelectrophoresis [31] using horse antiserum to whole human serum, precipitin arcs corresponding to IgG, albumin, IgA, IgM and other α- and β-globulins were visible. Four lots of fraction II were studied.

Fraction II which had been treated at pH 4 with pepsin 1:10,000 by the method of BARANDUN *et al.* [1] was supplied by the Central Laboratory of the Swiss Red Cross, Berne. Gelatin (Physiogel) and albumin (Cohn Fraction V analogue) were also Swiss Red Cross products. Albumin was defatted with charcoal at low pH [9].

Polymers and chemicals. Polyethylene glycols (PEG) with molecular weights of 1,000, 4,000 and 20,000 were practical grade from Fluka, Buchs, Switzerland. Polyvinyl pyrrolidone (PVP), methyl cellulose, 25 cP (MC), dextran and sodium heparinate (pure grade) and 1-octanol (analytical grade) were also from Fluka. Decane, olefin-free, *purum*, was obtained from Fluka. Dansyl chloride (1-dimethylaminonaphtalene-4-sulfonyl chloride, DNS Cl) was purchased from Calbiochem, Luzern, Switzerland. All other chemicals were analytical grade reagents from Merck, Darmstadt, FRG.

Glass beads. Glass beads (Ballotini) with average diameter of 0.9 mm and surface area of 3.8×10^{-2} cm² were treated with sulfuric acid saturated with potassium dichromate, and were thoroughly rinsed with twice-distilled water and air-dried before use. Beads were siliconized by five treatments with Siliclad (Clay Adams, Parsipany, N. J.) according to the manufacturers directions.

Preparation of purified IgG. For some experiments fraction II was further purified by DEAE-cellulose chromatography (Whatman DE 52) in 0.01 M sodium phosphate buffer, pH 7.8 [13]. The protein which emerged in the drop-through volume, representing about 80% of the applied protein, was pooled. This protein formed a single arc in the γ-region when tested at 30–40 mg/ml by micro-immunoelectrophoresis using horse anti-human serum and rabbit anti-human IgG. No IgA, IgM or albumin were detectable. In the text, this preparation is designated as IgG. Similar chromatographic conditions have been shown to remove a portion of IgG having fast-gamma electrophoretic mobility [13].

Exclusion chromatography on Sephadex G-150. Protein samples were fractionated on a column of Sephadex G-150 (5.0 × 85 cm) equilibrated in 0.02 M sodium phosphate buffer, pH 6.6, containing 0.13 M sodium chloride. The column was operated from top to bottom. Flow rate was maintained at 40 ml/h and 10-ml fractions were collected.

Protein concentrations. Protein content was determined by Kjeldahl analysis or by measurement of absorbance at 280 nm using 13.5 as the value for $A^{1cm}_{1\%}$ 280 nm for IgG.

Anticomplementary activity. Anticomplementary activities were assayed by the modified method of KABAT and MAYER [20] routinely used in the Swiss Red Cross. The diluent employed throughout was veronal-buffered saline (VBS), pH 7.3, containing optimum levels of Ca^{++} and Mg^{++} [20]. Sheep blood was collected in Alsever solution and stored for at least 1 week at +4°C. Fresh frozen guinea pig serum purchased from the Institute for General Microbiology, Bern, was used as the source of complement. Each lot was divided into 1-ml aliquots and stored at −30°C. Standardization of complement and sensitization of washed sheep erythrocytes were performed as recommended by KABAT and MAYER [20]. The complement levels in different lots of guinea pig serum ranged from 150 to 250 50-percent hemolytic units (CH50) per milliliter.

For titration of anticomplementary activity, two-fold serial dilutions (1 ml) of protein solution were made with VBS. 1 ml of diluted guinea pig serum containing 2 CH50 and 4.5 ml VBS were added to each dilution. Buffer blanks and complement controls containing 2 and 11 CH50 in a total volume of 6.5 ml were also prepared. The tubes were incubated at 37°C for 90 min. 1 ml of optimally sensitized erythrocyte suspension (5×10^8 cells/ml) was added and the samples were further incubated at 37°C for 60 min and cooled. The unlysed cells were removed by centrifugation and the optical densities of the supernatants were measured at 546 nm. Percent hemolysis was calculated relative to complement controls (2 CH50) taken as 100%. The anticomplementary titer was determined graphically as the exact dilution factor corresponding to 50% hemolysis. Specific anticomplementary activity, expressed as CH50/mg, is the reciprocal value of the weight of protein in this dilution.

Surface pressure. Surface pressure was measured by the WILHELMY [34] technique. A platinum plate with a perimeter of 39.6 mm, coated with platinum grey, was suspended from a transducing cell (Stratham, model UC2). A transducer/amplifier indicator (Hewlett-Packard, model 311A) amplified the signal which was recorded by a Philips 12-channel recorder. The teflon trough had dimensions of 10 × 10 × 1 cm, and was equipped with sweeping bars for cleaning the gas-liquid surface. Buffers used for the subphase were 0.02 M sodium phosphate, pH 7.0, containing 0.15 M sodium chloride and 0.02 M sodium acetate, pH 3.98, containing 0.15 M sodium chloride. Proteins and polymers were equilibrated in the buffer. An aliquot (10–200 μl) of solution to be tested was injected into the subphase through a hole in the frame of the trough. The subphase was stirred magnetically. The initial rate of increase of surface pressure ($d\pi/dt$) was calculated from the maximum slope of the recorded curve. The equilibrium surface pressure ($\Delta\pi$) was estimated as the asymptote of the curve. Several subphase concentrations of each substance were tested in duplicate.

Dansylation at decane-water interface. The procedure for dansylation at liquid-liquid interfaces was derived from the method of BROWNE *et al.* [5]. Solutions of DNS Cl (2×10^{-3} M) in decane were prepared immediately before use and were filtered to remove any insoluble dansyl hydroxide. 2.5 ml of this solution and 5.0 ml of protein solution were stirred magnetically for 30 min. The phases were separated by centrifugation. Aliquots of the aqueous solution or suspension were spotted on paper or were subjected to electrophoresis on cellulose acetate strips. The strips were dried and viewed under ultraviolet light to detect fluorescence of the protein bands.

Results

In the assay of the anticomplementary activity as described in *Methods*, the first step is the preparation of serial dilutions of the protein solution to be tested. In some of our experiments we noticed that relatively minor variations in the technique of serial dilution caused large changes in the anticomplementary titers of fraction II solution. For each curve in figure 1, twofold serial dilutions were made by pipetting 1 ml of protein solution into a tube containing 1 ml of buffer, mixing the contents and then transferring 1 ml to the next tube. The technique of mixing and transfer to the next tube were different in each of the three experiments. The 50-percent hemolytic titers for the respective curves were 1:41, 1:690 and 1:990, which correspond to specific anticomplementary activities of 0.6, 9.9 and 14.1 CH50/mg. When a rubber bulb was used to draw the protein solutions into the pipette for mixing solutions, the results were the same as with normal mouth pipetting. In all of the following experiments the dilutions were made with a single pipette and mixed by pipetting, as in curve 3, figure 1.

When fraction II was assayed for anticomplementary activity by the described procedure (fig. 2, curve 1), the 50-percent hemolytic titer was 1:775, corresponding to an activity of 13.0 CH50/mg. When this fraction II solution was serially diluted, and then albumin was added to each dilution, the results remained unchanged (curves 2 and 3). When albumin was added before the solution was serially diluted, the titer was 1:19, or 0.32 CH50/mg (curve 4). With different albumin concentrations (8 and 22 mg/ml), the activity was consistently 0.3 ± 0.05 CH50/mg. Some other substances were found to possess a similar stabilizing property. In further experiments, PEG 4,000 was also used instead of albumin; other compounds will be characterized later on.

If the anticomplementary activity of fraction II was measured in the presence of albumin concentrations below a certain level, biphasic hemolysis curves were obtained, as shown in figure 3. The activities of fraction II without albumin (curve 1) and with 5 mg/ml (curve 5) were 6.8 and 0.2 CH50/mg, respectively. In curve 2 (0.5 mg/ml of albumin) the concentration of albumin was sufficient to prevent the formation of any anticomplementary activity up to the 1:8 dilution. With further dilution, the hemolysis decreased to 0% at 1:64, and then increased. A similar dip was seen in the hemolysis curves obtained with higher initial concentrations of albumin, 1 and 2 mg/ml (curves 3 and 4). With 5 mg of albumin, no dip occurred.

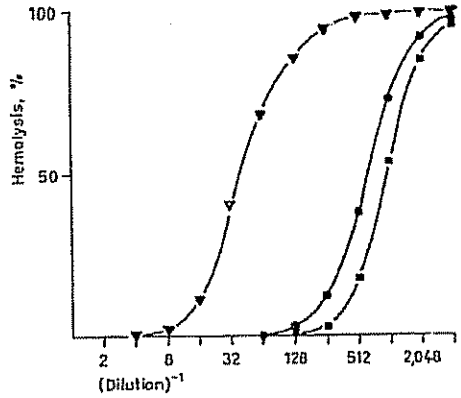

Fig. 1. Effect of different methods of serial dilution on the anticomplementary activity of fraction II. Two-fold serial dilutions (1 ml) of fraction II (70 mg/ml) were made with VBS diluent. Anticomplementary activities were measured as described in *Methods*. Curve 1 (▼): a separate 1-ml glass pipette was used for each serial transfer and the protein solution was mixed with VBS diluent by gently swirling the liquid in the tube; curve 2(●): separate pipettes were used and each dilution was mixed by pipetting four times; curve 3 (■): a single pipette was used throughout and mixing was done by pipetting four times.

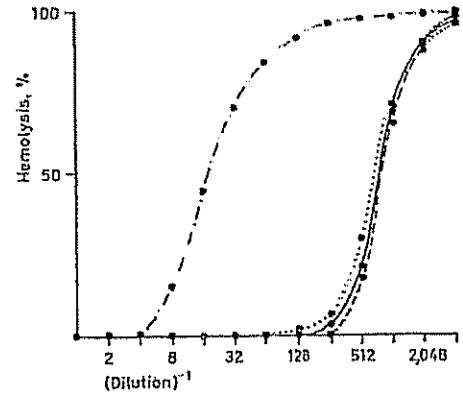

Fig. 2 Anticomplementary activity of fraction II without albumin and with albumin added before and after serial dilution. Curve 1 (——): fraction II (60 mg/ml) was serially diluted and assayed without albumin; curves 2 (. . .) and 3 (—): 1 ml of albumin solution, 2.2 mg/ml (curve 2) and 72 mg/ml (curve 3) were added to each dilution of fraction II; curve 4 (-·-·-): 1 ml of albumin solution (35 mg/ml) was added to 1 ml of fraction II solution (60 mg/ml) and the mixture was serially diluted and assayed.

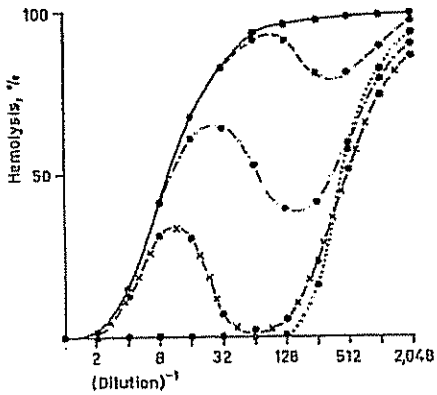

Fig. 3. Effect of different albumin concentrations on the anticomplementary activity of fraction II. Curve 1 (....): fraction II (35 mg/ml) was serially diluted and assayed without albumin; curves 2–5: Mixtures of fraction II (35 mg/ml) and albumin were serially diluted and assayed. Curve 2 (x-x-x): albumin, 0 5 mg/ml; curve 3 (- -): 1 mg/ml; curve 4 (—): 2 mg/ml, and curve 5 (——): 5 mg/ml.

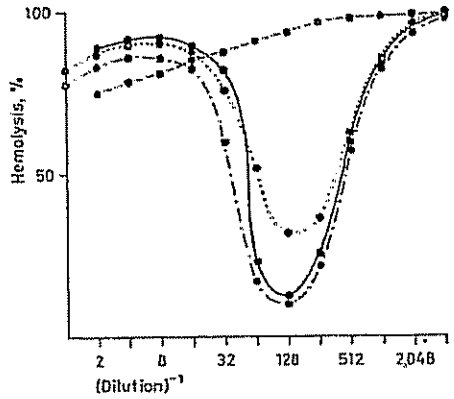

Fig. 4. Anticomplementary activity of pepsin (pH 4)-treated fraction II, assayed with and without albumin. Curve 1 (——): pH-4-treated fraction II (62 mg/ml) was serially diluted and assayed without albumin; curves 2(. . .) and 3 (- -): 1 ml of albumin solution 70 mg/ml (curve 2) and 200 mg/ml (curve 3) was added to each dilution of pH-4-treated fraction II; curve 4 (—): 1 ml of albumin solution (35 mg/ml) was mixed with 1 ml of pH-4-treated fraction II (62 mg/ml) before serial dilution.

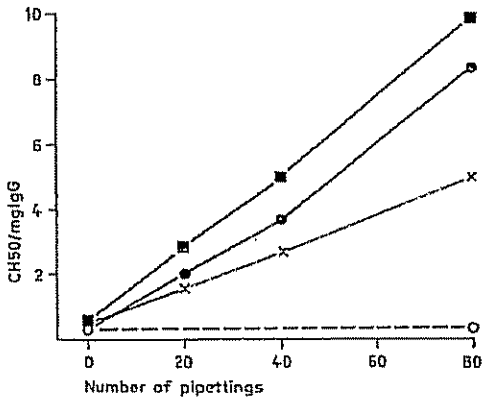

Fig. 5. Effect of repeated pipetting on the anticomplementary activity of fraction II. Aliquots of fraction II were pipetted with a 1-ml glass pipette 0, 20, 40 and 80 times. 1 ml of albumin solution (10 mg/ml) was then added to each sample and the anticomplementary activities were measured. Curves 1, 2 and 3 show the increase in anticomplementary activity with fraction II solutions of different concentrations. Curve 1 (■): IgG 2.2 mg/ml; curve 2 (●): 4.4 mg/ml; curve 3 (×): 17.5 mg/ml. Curve 4 (⊙) shows the activity of the same fraction II sample, 4.4 mg/ml; pipetted 80 times after the addition of albumin.

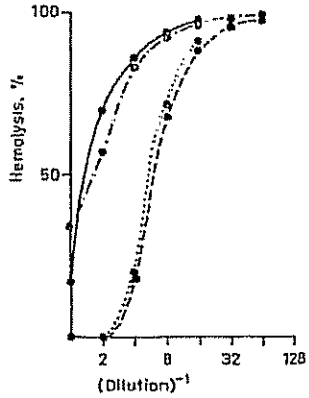

Fig. 6. Site of the anticomplementary activity formed by pipetting. 2-ml aliquots of fraction II (5 mg/ml) were tested as follows: Curve 1 (——): unpipetted; curve 2 (—): pipetted 10 times with a single pipette; curve 3 (- -): pipetted 10 times, each time with a separate pipette. PEG was added to the sample in curves 1–3 before serial dilution. Curve 4 (. . .): the solution remaining in the 10 pipettes from curve 3 was collected by rinsing with 2 ml of a solution of PEG in saline and assayed.

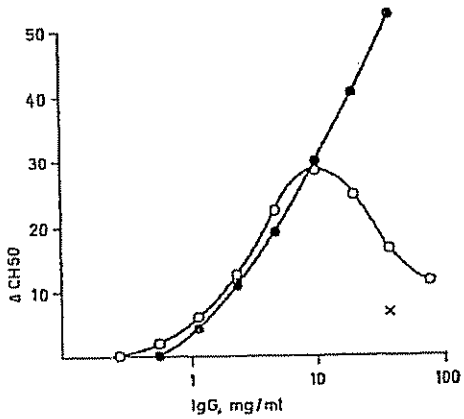

Fig. 7. Concentration dependence of the increase in anticomplementary activity with pipetting. Protein solutions were diluted to the designated concentrations. A 2-ml aliquot of each sample was pipetted 80 times. PEG was added and the activities were assayed. The increase in total anticomplementary activity was calculated as the reciprocal of the 50-percent hemolytic titer of the pipetted sample minus that of the corresponding unpipetted control. Curve 1 (⊙): fraction II; curve 2 (●): IgG. (A chromatographically purified sample of the same fraction II lot.) The activity of a mixture of IgG (37 mg/ml) and albumin (1 mg/ml) after 80 pipetting is designated by (×).

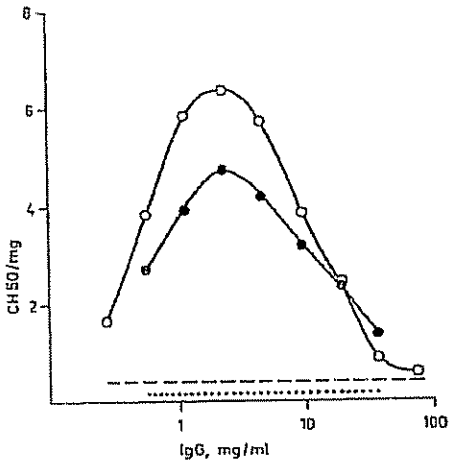

Fig. 8. Concentration dependence of the increase in specific anticomplementary activity (CH50/mg) with pipetting. The results in figure 7 are expressed as CH50/mg IgG. Curve 1 (⊙): fraction II; curve 2 (●): IgG. The dashed and dotted lines represent the activity (CH-50/mg) of unpipetted controls of Fraction II (—) and IgG (.)

When fraction II which had been treated at pH 4 with pepsin (1 : 10'000) was assayed, a similar biphasic hemolysis curve was obtained (fig. 4, curve 1). Curves 2 and 3 show that this effect was not reversed if albumin was added after serial dilution of the sample. When albumin was added to this preparation prior to serial dilution (curve 4), no dip in the hemolysis curve was observed at any dilution. Ten different pH 4–pepsin-treated fraction II preparations gave similar results. This effect has also been observed with fraction II preparations which had been treated with plasmin, β-propiolactone or at pH 4 without pepsin to reduce their anticomplementary activity.

Figure 5 shows the effect of repeated pipetting with a single glass pipette on the anticomplementary activity of fraction II solutions of different concentrations. 2-ml aliquots were pipetted 20, 40 or 80 times with a 1-ml pipette. Then albumin was added to each sample to prevent any further activity increase during the preparation of serial dilutions and the activity was assayed. As an unpipetted control, 2 ml of each solution was mixed with albumin and assayed for anticomplementary activity. Samples of fraction II solution which were mixed with albumin or with PEG and then pipetted 80 times had the same activity as the unpipetted controls. In the samples repeatedly pipetted before the addition of albumin, the anticomplementary activity increased in a nearly linear fashion. A linear increase resulted with each fraction II concentration, but the level of attained activity differed.

The results of the next experiment (fig. 6) show that the site of activity increase is in the film of protein solution remaining in the drained pipette. When fraction II solution was pipetted 10 times, each time with a different pipette, the anticomplementary activity of the solution was not increased over that of an unpipetted solution. The solution remaining in the 10 pipettes was collected in 2 ml of saline containing PEG, and was found to have the same activity as a solution which was pipetted 10 times with a single pipette.

In the following experiment, the increase in the anticomplementary activity of fraction II and of IgG, formed by pipetting solutions of different protein concentrations was studied (fig. 7). At concentrations up to 10 mg/ml, the anticomplementary activity of both samples was increased to approximately the same extent. At higher concentrations, the increase in the activity of fraction II was significantly less than that of the IgG. IgG mixed with a small amount of albumin (IgG 37 mg/ml, albumin 1 mg/ml) was only slightly increased in activity when pipetted 80 times.

In figure 8, the data of figure 7 are expressed as increases in specific anticomplementary activity (CH50/mg). According to the results when solutions

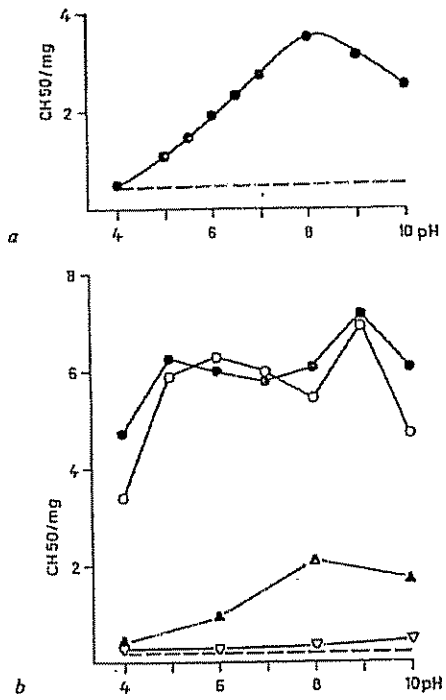

Fig. 9. pH dependence of the anticomplementary activity increase. *a* Solution of fraction II (18 mg/ml) were adjusted to the designated pH values. Two ml of each solution was pipetted 80 times, PEG was added and the solutions were neutralized and assayed (●). All samples were adjusted to the same concentrations before pipetting and after neutralization Activity of unpipetted solution (—). *b* Samples of IgG, with and without added albumin, were pipetted 80 times at the designated pH values. The samples were then stabilized, neutralized and assayed as in fig. 9a. Curves 1 (●) and 2 (⊙): two different IgG samples, 10 mg/ml; curve 3 (▲): a mixture of IgG (10 mg/ml) and albumin (0.1 mg/ml); curve 4 (▽): a mixture of IgG (10 mg/ml) and albumin (0.3 mg/ml). The same preparation of IgG was used for curves 1, 3 and 4. Activity of unpipetted IgG (—)

of fraction II or IgG of different concentrations are pipetted 80 times, the largest increase in specific activity occurs in solutions containing about 2 mg/ml of protein.

The dependence of the increase in anticomplementary activity of pipetted fraction II on pH is shown in figure 9a. It can be seen that at pH 4 the protein solution did not change its anticomplementary properties, and that maximum increase in activity occurred in the pH region near 8. When two different

IgG preparations were tested in the same way, the pH dependence was not pronounced as with fraction II: only a slight difference between acidic and alcaline region could be observed (fig. 9b). The IgG sample containing 0.1 mg/ml albumin was shown to be pH-dependent similarly to fraction II. The activity of the same IgG sample containing 0.3 mg/ml albumin was not changed significantly at any pH value.

The data in table I show that the anticomplementary activity caused by pipetting is in the form of aggregates, most of which are too large to pass through a 0.2-μm membrane. If the solutions were membrane-filtered immediately after pipetting, 90–100% of the increase in anticomplementary activity formed by pipetting was removed. Filtration 15–20 min after pipetting removed less of the activity, indicating that the aggregates were dissociated on standing. The anticomplementary activity of unpipetted control solutions was not decreased by filtration. A slight decrease in protein concentration occurred in all samples. All of the preparations had previously been passed through 0.2-μm filters as a sterilization step.

Elution patterns of unpipetted and pipetted solutions of fraction II obtained by exclusion chromatography on Sephadex G-150, showed that repeated pipetting did not significantly alter the elution characteristics of the protein solution (fig. 10). Shortly after elution, aliquots from the fractions containing the highest protein concentration in each of the three peaks were stabilized with PEG and assayed for anticomplementary activity. The results are shown in table II, column A. The data in table II, column B, show the anticomplementary activity of the same fractions pipetted 80 times, and then stabilized with PEG and assayed.

Various substances were found to have a similar action on the anticomplementary activity of IgG to that of albumin. Low titers were obtained in the presence of gelatine, PEG with molecular weights of 1,000, 4,000 and 20,000, PVP, MC and 1-octanol (serial dilutions were made with octanol-saturated VBS diluent). As observed with albumin, low levels of these substances resulted in biphasic hemolysis curves. In the presence of dextran, heparin, methyl cellosolve, ethylene glycol, ethanol or glycine, IgG exhibited the same high anticomplementary titers as when tested alone. None of the substances tested enhanced or inhibited hemolysis by complement controls or caused hemolysis of erythrocytes in the absence of complement. Freshly thawed solutions of different preparations of fraction II had similar anticomplementary activity when measured in the presence of different stabilizers. This activity tended to increase during storage at 4°C. Removal of impurities from fraction II by DEAE-cellulose chromatography generally resulted in a

Table I. Effect of membrane filtration on anticomplementary activity and protein content of unpipetted and pipetted IgG solutions

Protein solution tested	No. of pipettings per ml	Anticomplementary activity CH50/mg		Protein concentration mg/ml[1]	
		before filtration	after[2] filtration	before filtration	after[2] filtration
IgG	0	0.1	ND[3]	19.8	ND[3]
	40	10.8	1.0 (0 min)	19.9	18.2 (0 min)
			2.1 (15 min)		17.6 (15 min)
Fraction II	0	0.4	0.4	4.7	4.4
	40	4.8	0.4	4.7	4.4
Fraction II	0	0.7	0.7	4.7	4.5
	40	7.3	1.0 (0 min)	4.7	4.3 (0 min)
			1.4 (30 min)		4.4 (30 min)

[1] Protein concentrations were calculated from the mean value of two measurements of absorbency at 280 nm.

[2] Samples were membrane filtered immediately after pipetting, or 15 or 30 min later, where indicated.

[3] Not determined.

Table II. Anticomplementary activities of components I, II and III obtained by gel filtration of unpipetted and pipetted solutions of fraction II

Sample applied to Sephadex G-150	Component	CH50/mg	
		A	B
Unpipetted	I	3.0	18.5
Fraction II	II	0.2	10.2
Solution	III	0.2	6.2
Pipetted	I	10.8	40.2
Fraction II	II	0.2	14.2
Solution	III	0.2	9.0

Fractions representing the peak level of each component eluted from Sephadex G-150 (fig. 7) were tested shortly after elution. A 2.0-ml aliquot of the indicated fractions was mixed with PEG and assayed for anticomplementary activity (column A). Another aliquot was pipetted 80 times, mixed with PEG and then assayed (column B).

Table III. Increase in anticomplementary activity of IgG with various treatments

Treatment	Anticomplementary activity, CH50/mg		
	IgG	IgG+albumin	IgG+PEG
None	0.3	0.3	0.3
Pipetting 80 times	10.1	0.3	0.3
Bubbling with N_2, 40 min[1]	9.7	0.5	1.9
Stirring with decane, 40 min	36.5	4.3	8.1
Stirring with siliconized glass beads, 40 min	2.5	0.5	2.5
Stirring with glass beads, 40 min	0.6	0.9	0.5
Lyophilization	23.0	0.3	25.0

The IgG concentration in all solutions except those which were lyophilized was 5 mg/ml. In the second and third columns, albumin (10 mg/ml) or PEG (5 mg/ml) was added prior to treatment. In the solutions that were lyophilized, the concentration of IgG was 20 mg/ml; albumin 10 mg/ml and PEG 10 mg/ml.

[1] In 2 ml of protein solution N_2 was introduced through a teflon tubing (0.8 mm in diameter) with a speed of approximately 150–200 bubbles/min.

decrease in activity. This decrease may have been due to precipitation and removal of aggregates in the low ionic strength buffers used for dialysis and elution [19].

The striking increase in the anticomplementary activity of IgG occurs not only upon pipetting, but after various physical treatments as well (table III). The common feature of the first three treatments (pipetting, bubbling with nitrogen and stirring with decane) is the formation of interfaces of the air-liquid, liquid-liquid and solid-liquid type, and the exposure of the protein to these interfaces. During these treatments, the anticomplementary activity increased up to 100-fold. The treatments caused increased turbidity, and in some cases measurable loss of protein in the solution due to the precipitation. To compare the effects of hydrophobic and hydrophilic solid surfaces, siliconized and untreated glass beads (15 g, 180 cm^2 total surface area) were added to IgG solutions (6 ml, 5mg/ml) and slowly stirred for 40 min. The results (table III) indicate that more anticomplementary activity appeared

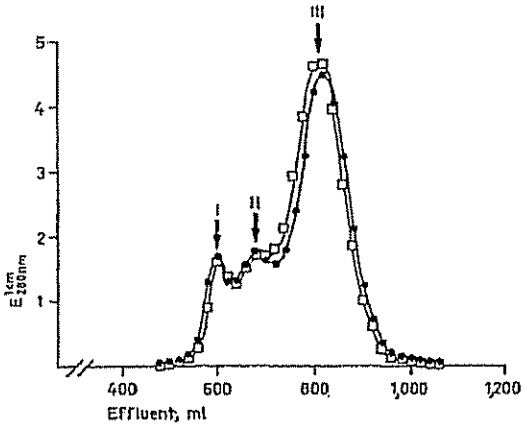

Fig. 10. Results of gel filtration of unpipetted and pipetted solutions of fraction II. Unpipetted (□) and repeatedly pipetted (●) solutions of fraction II (17 ml, 30 mg/ml) were applied to a 5 × 85 cm column of Sephadex G-150 equilibrated in 0.02 M sodium phosphate buffer, pH 6.6, containing 0.13 M sodium chloride. Flow rates were maintained at 40 ml/h and 10 ml samples were collected. The fractions designated I, II and III were taken for anticomplementary activity measurements shown in table III.

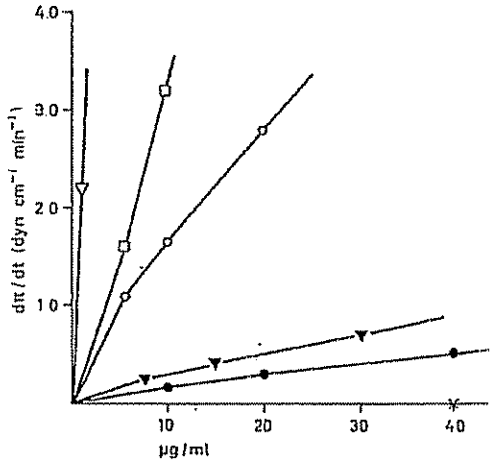

Fig. 11. Initial rate of increase in surface pressure caused by proteins and polymers. Aliquots of polymer solutions were injected into pH 7 subphase buffer in a Langmuir trough. The initial rate of surface pressure increase ($d\pi/dt$) was measured as described in *Methods*. ▽ = PEG; □ = PVP; ○ = albumin; ▼ = gelatin; ● = IgG; × = dextran, heparin

in the solution upon stirring with the hydrophobic than with the hydrophilic beads. In columns 2 and 3 the effects of these treatments on IgG solutions containing albumin or PEG are shown. Less anticomplementary activity was formed during these treatments. Albumin (column 2) was more effective than PEG (column 3) in preventing the increase in anticomplementary activity caused by the hydrophobic surfaces of nitrogen, decane and siliconized glass beads. However, albumin did not prevent the small increase in activity caused by hydrophilic glass beads. During lyophilization there was no protection by PEG, but a complete protection by albumin. In some treatments the protective effect of PVP was also tested. This polymer (5 mg/ml) was completely effective in preventing the change with pipetting, was equivalent to albumin in protecting IgG upon stirring with decane, and was less effective than albumin or PEG during the nitrogen treatment.

Previous experiments showed that IgG is stabilized during various physical treatments not only by albumin and PEG, but by some other polymeric substances as PVP and gelatin; dextran and heparin were not effective. The data in figure 11 show the rates of increase in surface pressure at the air-liquid interface after solutions of these polymers were injected into a buffer subphase in a Langmuir trough. The rate of surface pressure increase ($d\pi/dt$, dyn cm^{-1} min^{-1}) can be considered an indication of the rate of accumulation of the substance at the interface. PEG, PVP, albumin and gelatin caused the surface pressure to increase more rapidly than did IgG over a range of concentrations (1–40 μg/ml). The equilibrium surface pressures for these substances at 10 μg/ml were: PEG, 5.9 dyn/cm; PVP, 3.5 dyn/cm; albumin, 12.5 dyn/cm; IgG, 5.5 dyn/cm. The equilibrium surface pressure of gelatin was not determined. Dextran and heparin did not cause any measurable change in the surface pressure.

In one of the previous experiments, the increase in anticomplementary activity of IgG with pipetting was shown to be less dependent of pH, than when albumin was present in the solution (fig. 9). At pH 4, albumin was more effective in preventing the increase in the activity. When various concentrations of IgG were injected into a pH 4 subphase buffer in the Langmuir trough, the rate of surface pressure increase was found to be nearly equal to that measured at pH 7. The rate of pressure increase caused by albumin at pH 4 was approximately 6 times faster than at pH 7. The equilibrium surface pressures of albumin and IgG were not changed at pH 4.

CECIL and LEWIS [8] reported that insulin was readily adsorbed at the surface of decane emulsion droplets. By incorporation of the hydrophobic protein reagent DNS Cl into the decane phase, BROWNE *et al.* [5] succeeded

Table IV. Fluorescence of proteins dansylated at decane-water interface

Composition of aqueous phase mg/ml		Fluorescence of aqueous phase after emulsification with solution of dansyl chloride in decane				
IgG	albumin	spot test		acetate electrophoresis		
		soluble	precipitate	albumin	IgG	precipitate
10	0	–	+++	–	–	+++
10	10	+++	–	+++	–	–
10	2	+++	–	+++	–	–
10	0.5	+++	–	++	–	–
10	0.1	++	±	±	–	±
0	0	–	–	–	–	–
0	10	+++	–	+++	–	–

For each experiment, 5 ml of protein solution or buffer was emulsified for 30 min with 2.5 ml of the hydrocarbon phase consisting of a solution of 2×10^{-3} M DNS Cl in decane. The phases were separated by centrifugation, and aliquots of the aqueous phase solution or suspension were tested by spoting on paper or by electrophoresis in cellulose acetate strips. Fluorescence of precipitated or soluble proteins was detected under UV light. +++=Strong fluorescence or precipitation; ++=moderate fluorescence or precipitation; +=slight fluorescence or precipitation; ±=very slight fluorescence or precipitation; –=no detectable fluorescence or precipitation.

in attaching a fluorescent label to the portion of the insulin molecule in contact with the hydrophobic phase. DNS Cl is poorly soluble in water and remains partitioned in the decane phase, where it is able to react only with residues in the interfacial region. This approach can also be used with aqueous solutions of more than one protein to determine if preferential adsorption of one protein occurs. The data in table IV show the results of preliminary experiments in which a solution of DNS Cl was emulsified in aqueous solutions of IgG and albumin. When IgG solutions were vigorously stirred with hydrophobic marker phase for 30 min, a portion of the protein became insoluble and highly fluorescent. The soluble protein did not show a visible fluorescence. When solutions containing IgG (10 mg/ml) and albumin (0.5, 1.0 and 10 mg/ml) were emulsified with DNS Cl solution, the aqueous phase became strongly fluorescent and no precipitate formed. After separation of the proteins by electrophoresis, only the albumin band

was highly fluorescent. With 0.1 mg/ml albumin and 10 mg/ml IgG, in addition to the labeled albumin some fluorescent precipitate formed. PEG also prevented the formation of fluorescent IgG precipitates, but did not prevent albumin from being labeled.

Discussion

MALGRAS *et al.* [24] previously reported that serial dilution could increase the anticomplementary activity of IgG solutions. In the present investigation we also found that different techniques of preparing serial dilutions of the same fraction II solution resulted in a wide range in the measured values for anticomplementary activity. Because the three assays in figure 1 were carried out identically except for the way in which the dilutions were mixed, the observed difference can only be explained as an increase in activity caused by pipetting. The data in figure 5 confirm these findings. Repeated pipetting before the assay is shown to cause a nearly linear increase in the anticomplementary activity of fraction II solutions. The same results were obtained with rubber bulb and with normal mouth pipetting, indicating that the increased anticomplementary activity is not due to contamination with antigens in the breath and formation of immune complexes with antibodies in the fraction II preparation. Pipetting *per se* must cause some changes of IgG molecules in solution to render them anticomplementary. Increased turbidity of the pipetted protein solutions first suggested that it might be aggregation. The results obtained when pipetted IgG solutions were membrane filtered were in agreement with this observation (table II). Because the anticomplementary activity formed by pipetting was almost completely removed and no significant decrease in the protein concentration could be detected, it is probable that small amounts of very large aggregates are responsible for the increased activity. Gel filtration experiments are in agreement with these results. Although no increase in the extent of aggregation could be seen in elution pattern of the pipetted sample (fig. 10), the activity of the excluded component which was respinsable for most of the anticomplementary activity, was increased (table II).

In some of the experiments, it was shown that albumin can prevent the increase in anticomplementary activity of fraction II caused by pipetting during (fig. 2) or before (fig. 5) the assay for this activity. Some investigators have already reported similar observations but the phenomenon remained unclarified. DAVIS *et al.* [11] found highanticomplementary titers for IgG

which had been isolated from serum by electrophoresis or by precipitation with sodium sulfate. When albumin or α- and β-globulins were added, the titers decreased eightfold or more. They suggested that these serum proteins might act by interfering with or reversing the interaction of IgG and complement. BECH [2] also observed that IgG became much less anticomplementary when incubated with albumin, human serum or animal serum. The results of DAVIS *et al.* [11] and of BECH [2] were both obtained with assayas in which the IgG solution was serially diluted. The low titers they observed in the presence of albumin or other serum proteins are consistent with the present observations and probably represent stabilization of IgG. The results in figure 2 (curves 2 and 3) show that albumin does not interfere with complement fixation.

Biphasic hemolysis curves were obtained when anticomplementary activity of fraction II was assayed in the presence of albumin concentrations below 5 mg/ml (fig. 3). Each curve appears to dip at the dilution where albumin level reached a minimum concentration necessary for complete stabilization of IgG under the experimental conditions (concentration of IgG, number of pipettings, number of serial dilutions). This level is in the range of 0.03–0.25 mg/ml for defatted albumin. Dips in hemolysis curves observed with pepsin (pH 4)-treated fraction II (fig. 4) indicate the presence of stabilizing factor(s) in these preparations. Some preliminary experiments indicated that these are the traces of IgG pepsin fragments and albumin.

In addition to albumin a variety of other substances was found to protect IgG against the alteration caused by pipetting. The values for the anticomplementary activity of a given IgG preparation measured in the presence of proteins (albumin, gelatin) or several synthetic polymers (PEG, PVP, MC) were in close agreement. Polysaccharides (dextran, heparin) did not protect IgG, but a partially methylated polysaccharide (MC) was a very effective stabilizer. Glycine, commonly used as a stabilizer of IgG solutions [15, 17, 23], did not protect IgG during serial dilutions. Of the low-molecular-weight substances tested, only 1-octanol was effective.

Buffers containing albumin or gelatin (0.1%) have been used in complement fixation test to prevent nonspecific hemolysis of erythrocytes [20] and adsorption of complement [7, 14] or protein antigens [26] to glass. The present results indicate that albumin or gelatin can also be used to prevent increase in anticomplementary activity when IgG solutions are pipetted. In preliminary experiments where 0.1% albumin or gelatin were added to the dilution buffer anticomplementary titers were two to six times higher than when albumin or gelatin were added to the initial IgG solution. The activity

may have been increased in the first serial transfer of IgG into the buffered albumin or gelatin solution. No stabilizer was present at this step.

Various physical treatments were found to cause the alteration of IgG molecules in solution. The anticomplementary activity was shown to be markedly elevated by exposure to surfaces of the gas-liquid, liquid-liquid and solid-liquid type (table III). It is well established that IgG is readily adsorbed to various types of surfaces [4]. When adsorbed to polystyrene [3, 27] and to bentonite [25], IgG increased anticomplementary activity. The adsorption of IgG to polystyrene is prevented if the surface is first exposed to albumin [3], gelatin [29], serum [3] or detergents [29]

The results obtained when fraction II solution was repeatedly pipetted each time with a separate pipette (fig. 6), suggest that the anticomplementary activity increase caused by pipetting is also due to exposure to interfaces. The solution adhering to the inner wall of the pipette is exposed to relatively large gas-liquid and glass-liquid interfaces. Because the anticomplementary material appeared in the liquid remaining in the pipette and not in the pipetted fraction II solution, it seems probable that the gas-liquid interface is the site of the activity increase with pipetting. With each successive pipetting, protein adsorbed at this interface is returned to the solution and a new gas-liquid interface is formed. The formation of the similar amount of anticomplementary activity with each pipetting is consistent with the linear increase in activity shown in figure 5.

KOCHWA *et al.* [22] have demonstrated that IgG is unfolded upon adsorption to polystyrene latex and that the extent of unfolding depends on the concentration of IgG at the surface. We found that the alteration of IgG caused by pipetting also depended on the concentration of the protein in solution. The increase in specific anticomplementary activity showed a sharp maximum near 2 mg/ml (fig. 8). This may represent the concentration at which the highest proportion of molecules are aggregated. Alternatively, the conformation or special arrangement of IgG molecules in aggregated form may be optimal for complement fixation. The difference in the total activity increase between fraction II and IgG (fig. 7) is probably due to the contaminant proteins which stabilize fraction II in concentrated solutions.

A good correlation was found between the ability of a given substance to protect IgG and the rate at which it accumulated at the air-liquid interface. Polymers with stabilizing ability (PEG, PVP, albumin and gelatin) accumulated at the air-liquid interface more rapidly than IgG and those which did not protect IgG (dextran and heparin) were not measureably adsorbed (fig. 11). The protection during other treatments in which IgG is

in prolonged contact with surfaces may depend on other characteristics such as rate of desorption, compactness of packing at the interface, equilibrium surface pressure, etc. PEG and PVP were more rapidly adsorbed than albumin but were less effective than albumin in preventing the aggregation of IgG with most treatments (table III). The equilibrium surface pressure of PEG and PVP were significantly lower than that of albumin.

No significant difference was found in the rate of adsorption of IgG at the air-liquid interface at pH 4 and at pH 7. This is consistent with the finding that IgG did not show a pronounced difference in the anticomplementary activity increase, when pipetted at different pH values. VAN OSS and SINGER [29] previously reported that the adsorption of IgG to polystyrene surfaces is independent of pH. This suggests that hydrophobic rather than ionic interactions are involved in adsorption at interfaces and in aggregate formation with pipetting.

A marked pH dependence was observed in the aggregation of IgG in the presence of albumin (fig. 9). The rate of adsorption of albumin at the air-liquid interface was found to be six times faster at pH 4 than at pH 7. BULL [6] showed that the adsorption of albumin to glass was maximum at pH 4–5. The increased surface activity of albumin is a possible explanation for its greater stabilizing activity at low pH values.

In a further attempt to clarify the mechanism of the protection of IgG by albumin, we used solutions of DNS Cl in decane to attach the fluorescent label to the interfacially adsorbed protein. Preliminary experiments showed that IgG was precipitated and labeled when solutions were emulsified with DNS Cl/decane solutions (table IV). The presence of albumin or PEG prevented both the precipitation and labeling of IgG at the interface. Neither IgG nor PEG prevented the labeling of albumin. These results suggest that albumin was selectively adsorbed from the mixtures and interfered with the adsorption, labeling and aggregation of IgG.

BERNHARD *et al.* [3] adsorbed an IgG solution repeatedly with polystyrene latex and found that less protein was adsorbed with each successive treatment. They interpreted this to mean that IgG was heterogeneous in its adsorption to the hydrophobic surface. PARKER and OSTERLAND [30] showed that human IgG myelomas differed in their ability to interact with hydrophobic probes. The hydrophobic binding sites were located in the Fab region and were probably at or near the exterior of the molecule. An extensive study of a series of well-defined myeloma proteins which is in progress, may show if surface behavior and aggregation are related to structural characteristics of a specific region of the IgG molecule.

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